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Chemical constituents, antimicrobial and antioxidant activity of essential oil of *Citrus limetta* var. Mitha (sweet lime) peel in Pakistan

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The essential peel oil of *Citrus limetta* var. Mitha (Sweet lime) extracted by hydro-distillation was assessed for chemical constituents, antimicrobial and antioxidant activity. Gas chromatographic analysis identified 17 constituents among which limonene (95.98 %) was found as major component followed by camphene (1.79 %), while the remaining terpenes were less than 1%. The results of antimicrobial activity of essential oil tested by disc diffusion method, against different against bacteria and fungi showed that it exhibited maximum zone of inhibition against *Bacillus cereus* ATCC 14579 (28 mm) and *Bacillus subtilis* ATCC 6633 (26 mm) followed by *Staphylococcus aureus* ATCC 25923 (21 mm), where as the minimum zone of inhibition was shown by *Fusarium oxysporum* ATCC 48122 (11 mm) after 48 h of incubation at their respective temperature (37°C for bacteria and 25°C for fungi). The inhibition zones, measured after 48 and 96 h, showed that it was active against all tested bacteria and fungi. The results of its antioxidant activity showed that it was able to reduce the stable radical 1-diphenyl-2-picrylhydrazyl (DPPH) to yellow-colored DPPH-H reaching 87.77% of DPPH scavenging effect at its 100% concentration comparative to ascorbic acid as reference standard being a strong antioxidant reagent. The results of our study showed that essential oil of *C. limetta* var. Mitha peel can be an effective medicine against different pathogenic microbes.

Key words: *Citrus limetta* var. Mitha, essential oil, antimicrobial activity, antioxidant, pathogenic microbes.

INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases because they contain chemical components of therapeutic value (Nostro et al., 2000). According to the World Health Organization (WHO) in 2008, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Pierangeli, et al., 2009). The genus citrus (Rutaceae) comprises of trees, shrubs and herbs of various sizes and uses. They are the most widespread arboreal plants in the world and represent one of the most important crops (Jacquemond et al., 2002). Citrus fruits belong to six genera (*Fortunella*, *Eremocitrus*, *Clymendia*, *Poncirus*, *Microcitrus* and *Citrus*), which are native to the tropical and subtropical regions of Asia, but the major commercial fruits belong to genus *Citrus*. The genus citrus is represented by 10 species in Pakistan (Nasir and Ali, 1978). *Citrus limetta* var. Mitha (sweet lime) is one of them. It is a popular indigenous citrus fruit

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relished for its cooling and therapeutic effects and also as a culinary delight in some parts of the subcontinent. In the traditional indigenous medicinal system, sweet lime juice is valued for curing fever, malaria and jaundice (Murphy, 1999).

One of the important products of citrus fruits is the essential oil, which is obtained from citrus peels (Mondello et al., 2005). Scientists from divergent fields are investigating the essential oils of different plants with an eye to their medicinal usefulness. These oils are considered to be one of the potential sources for the screening of anticancer, antimicrobial, antioxidant, and free radical scavenging agents (Shabbir et al., 2009). Phenolic compounds present in essential oils have been recognized as the bioactive components for the antimicrobial activity. Most plant phenolic compounds are classified as generally recognized as safe (GRAS) substances; therefore they could be used to prevent growth of many food-borne and food spoilage micro-organisms in foods. Such natural compounds are believed to demonstrate anticarcinogenic potential and offer diverse health-promoting effects because of their antioxidant attributes (Liu et al., 2008). The essential oils possessed appreciable antioxidant and radical scavenging activities revealing potential for therapeutic applications (Hussain et al., 2011). Essential oils and their components are gaining interest because of their relatively safe status, their wide acceptance by consumers and their exploitation for potential multi-purpose functional use.

Citrus essential oils are a mixture of volatile compounds and mainly consisted of monoterpene hydrocarbons (Sawamura et al., 2004). It is well known that essential oils from *Citrus* spp. have pronounced antimicrobial effect against both bacteria and fungi. Citrus essential oils could represent good candidates to improve the shelf life and the safety of minimally processed fruits (Lanciotti et al., 2004). Citrus essential oils are used in pharmaceutical, perfumery and food industries (Norajit et al., 2007). Moreover, increase in the emergence of new bacterial strains that are multi-resistant coupled with the non-availability and the high cost of new generation antibiotics have resulted in increase morbidity and mortality (Lewis and Ausubel, 2006). So, investigations into the antimicrobial activities, mode of action and potential uses of plant essential oils have regained momentum. As essential oils are a rich source of biologically active compounds, there has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils.

Citrus oils of different species have been studied (Vlisidis and Israilidis, 1998; Kirbaslar and Kirbaslar, 2003). However, essential oil of *C. limetta* var. Mitha (a local variety) in Pakistan has yet not been studied. The aim of the present studies was to determine the chemical composition, antioxidant and antimicrobial activity of essential oil of *C. limetta* var. Mitha cultivated in Pakistan against different food borne pathogens of public health significance.

MATERIALS AND METHODS

Collection of plant materials and extraction of essential oil:

The fruit was purchased from the local market of Lahore then washed, peeled off and cut into small pieces. The cut peels (300 gm) were subjected to Hydro-distillation by using Dean-Stark assembly (Sattar, 1989). The distillate was removed and separated from water by using a separating funnel. 0.939 g of oil is collected and extracted twice with 20 ml ether and was dried over 0.01 g anhydrous sodium sulphate and recovered pale yellow oil was stored for further studies.

Gas chromatographic analysis

Gas chromatographic analysis of the extracted peel oil was carried out on gas chromatograph (GC) of Agilent Technologies Inc., USA, operating in electron ionization mode at 70 eV using co-injection techniques in order to identify its different components. A 0.2 µL sample of the oil was injected and column was run under following conditions: Column, SE30 packed glass column; carrier gas, helium; flow rate, 30 ml/min; detector, C-R4A (Shimadzu); chart speed, 3 cm/min; column temperature, 70°C/min- 5°C/min. Various components were identified by their retention time and confirmed by injecting authentic standard samples. The percentage composition of the essential oil was obtained with the help of linked computing integrator (Shimadzu C-R4A).

Antibacterial and antifungal activities of *C. limetta* var. Mitha peel oil

The agar disc diffusion method was employed for the determination of antibacterial and antifungal activity of *C. limetta* peel oil following the procedure of Baydar et al. (2004) against different food borne pathogens including bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579, *Lactobacillus acidophilus* ATCC 4356 , *Escherichia coli* ATCC 25922, *Salmonella typhymurium* ATCC 14028 *and Enterobacter areogene* ATCC 13048) and fungi (*Aspergillus niger* ATCC 16404, *Aspergillus flavus* ATCC 204304, *Aspergillus fumigatus* KM 8001, *Aspergillus ficuum* ATCC 66876, *Aspergillus oryzae* ATCC 10124, *Fusarium oxysporum* ATCC 48122, *Penicillum digitatum* ATCC 201167, *Fusarium miniformes* MAY 3629, *Fusarium saloni* MAY 3636., *Candida utilis* ATCC 9950). Standard culture media (CM139, CM271, CM145, CM69, CM7 and CM201) from Oxoid were employed through-out the present investigation for the purpose of culture maintenance at their respective temperatures that is 25°C for fungi and 37 and 30°C for bacteria. *A. niger* and *A. flavus* from Guava fruit, *A. fumigatus* from Stored citrus fruit, *A. ficuum* from Lemon, *F. oxysporium* from Onion, *C. utilus* and *P. digitatum* from Bread, *S. aureus* and *L. acidophilus* from milk, *B. subtilis* and *B. cereus* from cheese, *E. coli* from *Aloe vera gel, S. typhymurium* from cake mix sample and *E. aerogenes* from baby food origin were obtained.

The growth medium were prepared, autoclaved and transferred aseptically to sterilized petri plates. Microbial cultures which were maintained in test tube slants were transferred to their respective media petri plates. Sterile and dried 6 mm paper discs (Difco, USA) were impregnated with 20 µl filtered sterilized (0.45 mm Millipore filter) newly extracted *C. limetta* var. Mitha peel oil. These oil-impregnated discs were dried under laminar flow cabinet. The

Table 1. Chemical composition of essential oil of *Citrus limetta* var. Mitha (sweet lime) by gas chromatography.

Component	Peak Number	Retention time (min)	Peak area	Percentage
α-thujene	1	1.354	5301	0.0688
α-pinene	2	1.483	2927	0.0381
β-pinene	3	2.006	2306	0.0299
ρ-cymene	5	4.933	29758	0.3861
Camphene	6	5.924	137681	1.7865
Limonene	7	7.468	7396614	95.9768
α-terpinene	8	8.658	25714	0.3337
Neral	9	9.967	22405	0.2907
Geraniol	10	11.273	28059	0.3641
Geranial	11	11.833	2427	0.0315
Citronellal	12	12.429	7329	0.0951
α-terpinol	13	13.171	24586	0.3191
Un-known	14	14.671	4246	0.0551
Un-known	15	15.563	1492	0.0193
α-humulene	17	18.263	3931	0.0511
β-bisabolene	19	19.971	9834	0.1276
β-sinesol	20	21.484	2062	0.0268

discs were placed on freshly seeded microbial lawns (4 discs in each plate) with a control. 20 µl of sterile water was used as a negative control, whereas streptomycin (20 µl/disc) as a positive control for bacteria and fluconazole (20 µl/disc) for fungi. All experiments were conducted in triplicate. The petri plates were incubated at their respective temperatures and zones of inhibition thus developed against tested microorganisms were measured in millimeters after a period of 24 h for yeast (*C. utilis*) and bacteria and 48 h for other fungi and similarly at 48 and 96 h (Table 2). The results of antimicrobial activity of peel oil against different micro-organisms were expressed as resistant, intermediate and sensitive.

Antioxidant activity of *C. limetta* var. Mitha peels oil

Antiradical activity was evaluated by measuring the scavenging activity of the examined *C. limetta* var. Mitha peel oil on the 2, 2-diphenyl-1-picrylhydrazil (DPPH) radical. The DPPH assay was performed as described by Epsin et al. (2000). The samples (100 µl each) were mixed with 3 ml of DPPH solution. The absorbance of the resulting solutions and the blank (with only DPPH and no sample) were recorded after an incubation time of 30 min at room temperature against ascorbic acid as a positive control. For each sample, three replicates were recorded. The disappearance of DPPH was measured spectrophotometrically at 517 nm. The percentage of radical scavenging activity was calculated using the following equation

DPPH scavenging effect (%) = $(A_0 - A_1)/A_0 \times 100$

Where, A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min.

RESULTS AND DISCUSSION

Chemical analysis of *Citrus limetta* var. Mitha peel oil

Hydro distillation of the peels of *C. limetta* var. Mitha yielded 0.313% oil. Yield of citrus essential oil differs with individual plant species ranging in most cases from 0.2 to 2.0% (Anonymous, 2004). Ahmad et al. (2006) studies showed that oil yield of Mousami, Grape fruit, Kinnow and Fewtrell's was 0.98, 0.73, 0.32 and 0.22%, respectively. This difference in the percentage yield of peel oil might be due to seasonal variation or time of harvest of citrus fruit.

Gas chromatographic analysis revealed that limonene which is the chief constituent in about all the citrus oils was the largest single monoterpene hydrocarbon (95.97%) of the *C. limetta* var. Mitha peel oil (Table 1). Limonene is also present, though in slightly lower quantities, in the oils of other major fruits, where it ranges from 85-95%. Similarly, Turkish lemon peel oil and sweet orange have high content of monoterpene hydrocarbons (89.9%) with limonene (61.8 and 91.6%, respectively) as major constituent (Kirbaslar et al., 2009). Tao et al. (2009) also studied the GC/MS analysis of essential peel oil of Bingtang sweet orange (*Citrus sinensis Osbeck*) and found that limonene was observed dominant (77.49%), followed by myrcene (6.27%), α-farnesene (3.64%), γ-terpinene (3.34%), *α*-pinene (1.49%), sabinene (1.29%) and other minor components. Scientists from different fields carried out comparative studies on the chemical composition of essential oils taken from Italian sweet lime and bergamot oil and found that both contained similar composition but the former had more limonene and less linalyl acetate content (Carpano et al., 2003; Haristoy et al., 2003). The second major constituent in our studied peel oil after limonene was camphene 1.78% followed by ρ-cymene 0.38%, Geraniol 0.36%, α-terpinene 0.33%, α-terpineol 0.31%, Neral 0.29%, β-bisabolene 0.12%. Similarly, it was found

Table 2. Anti-microbial activity of essential oil of *Citrus limetta* var. Mitha (Sweet Lime) peel against different pathogens.

Tested Microorganism	Incubation Temperature (°C) and Culture media	Inhibition Zone (mm)		Percentage decrease in inhibition zone (mm) after 96 h	Positive control Streptomycin/ Fluconazole (20µl/disc)	Negative Control	Efficiency
		After 24h (bacteria and yeast) 48 h for fungi	After 48 h (bacteria and yeast) 96h for fungi				
Aspergillus niger	25 & M139	22	17	26.22	20	0	S
Aspergillus flavus	25 & M139	19	16	18.97	12	0	I
Aspergillus fumigatus	25 & M139	14	12	15.71	10	0	I
Aspergillus ficuum	25 & M139	12	10	16.67	8	0	I
Fusarium oxysporium	25 & M139	11	10	10.90	9	0	R
Candida utilus	25 & M139	13	11	16.92	11	0	I
Pencillium digitatum	25 & M139	18	15	17.98	17	0	I
Staphylococcus aureus	37 & M145	21	18	16.82	20	0	I
Bacillus subtilis	37 & M271	26	22	21.13	24	0	S
Bacillus cereus	37 & M271	28	21	27.46	27	0	S
Lactobacillus acidophilus	45 & M361	18	16	14.75	14	0	I

that citronellal, geranial, α-pinene, β-pinene, α-thujene, β-sinesol and α-humulene were present in minute amount (less than 0.1%).

Our results were in conformity with the previous findings as limonene, γ- terpinene, β-pinene, α-pinene, myrcene, valencene, linalool, octanal, decanal, and butyle butyrate has been found as the major constituents through gas chromate-graphic separation of essential oils from vietnames pummelo, sweet orange, tangerine, bergamote, grapefruit peel and mousami (Gancel et al., 2002; Hognadottir and Russell, 2003; Khanum et al., 2004). Steam-distilled volatile peel oil of Indian orange when analyzed through GC and GC-MS, limonene was found more dominant followed by myrcene, α-terpinolene and β-pinene (Kirbaslar and Kirbaslar, 2003). Almost similar results were reported by Feger et al. (2003) and Tu et al. (2003) while working on orange and tangerine essential oils. Comparatively, however, the gas chromatographic separation of the citrus peel oils of Pakistani origin showed both quantitative and qualitative variation in volatiles composition. Chemical composition of essential oils of different species of citrus varied significantly, which may be due to the difference in their genetic makeup (Ahmad et al., 2006).

Antibacterial and antifungal activities of C. limetta var. Mitha peel oil

The results of antibacterial and antifungal activity of *C. limetta* var. Mitha peel oil, investigated against different food borne pathogens by disc diffusion method, are presented in Table 2. It was found in the present study that peel oil exhibited maximum zone of inhibition against B. cereus ATCC 14579 (28 mm) and B. subtilis ATCC 6633 (26 mm) followed by S. aureus ATCC 25923 (21 mm) after 48 h of incubation at 37°C, whereas the minimum zone of inhibition was shown by F. oxysporum ATCC 48122 (11 mm) after 48 h of incubation at 25°C in comparison with streptomycin/fluconazole at 20 µl per disc. However, *A. niger* ATCC 16404, *A. flavis* ATCC 204304, *A. fumigates* KM 8001, *A. ficuum* ATCC 66876, *C. utilus* ATCC 9950, *P. digitatum* ATCC 201167, E. coli ATCC 25922, *L. acidophilus* ATCC 4356, *S. typhymurium* ATCC 14028 and E. aerogenes ATCC 13048 gave 22, 19, 14, 12, 13, 18, 13, 18, 17 and 13 mm of zone of inhibition,

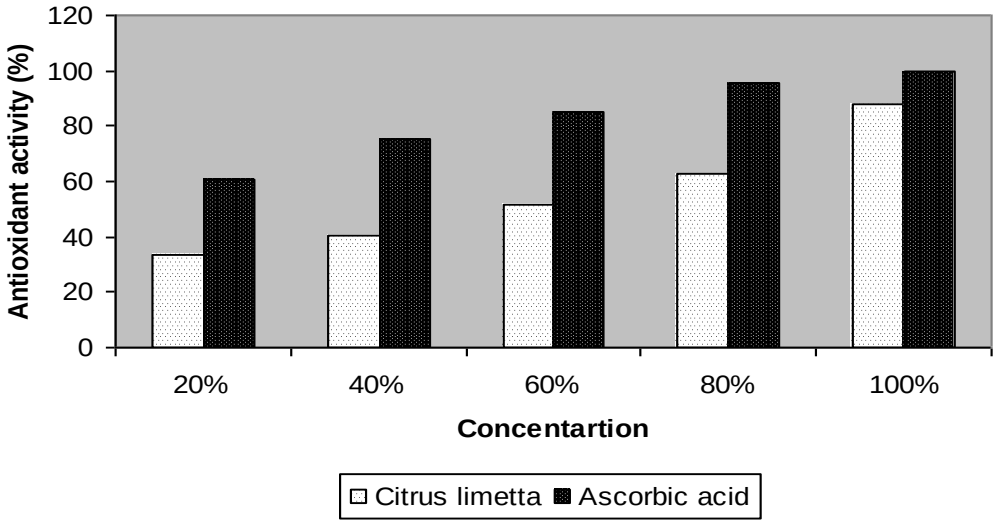

Figure 1. Percentage antioxidant activity of essential oil of *Citrus limetta* var. Mitha (sweet lime) in comparison with ascorbic acid as standard reference evaluated by DPPH assay.

respectively after 48 h of their respective incubation temperatures. The essential oils from two cultivars of tropical citrus, including *C. hystrix* and *C. aurantifolia* exhibited antimicrobial activity against *B. cereus*, *S. aureus* and *S. typhi* (Chaisawadi et al., 2003). Mahmud et al. (2009) results also revealed that *C. acida* peel oil exhibited maximum zone of inhibition against *B. subtilis*. Many other studies also revealed that α--pinene, limonene and linalool have a strong antibacterial activity (Mecciaa et al., 2007; Filipowicz et al., 2003; Koji et al., 2004). Kekuda et al. (2009) studied antifungal activity of steam distillates of peels of different citrus fruits namely *C. limetta*, *C. sinensis* and *C. limon* against *Aspergillus* species and found that *C. limetta* was found to be superior to inhibit the growth of tested fungi. Johann et al. (2007) studies also indicated that the peels of the Citrus species present substantial antimicrobial properties. Essential oils usually occur as complex mixtures and their activity can generally be accounted for in terms of their major monoterpenoid components. According to Sikkema et al. (1995), the antimicrobial action of monoterpenes suggests that they can easily diffuse into or penetrate through the damage cell membrane structures of microorganisms. Therefore, essential oils being rich in terpenes have been shown to possess good antibacterial activity (Afolayan and Ashafa, 2006). It is further reported that the antimicrobial property of the essential oil is because of the fact that it contains active components which influence certain metabolic functions of microbial cells (Chaisawadi et al., 2003). In addition, some components that occur in lesser amount may also contribute to the antimicrobial activity of the oil, involving probably some type of synergism with the other active compounds (Matosyoh et al., 2007). It was also found during the present investigation that the tested oil has shown nearly equal antimicrobial effects on both gram positive (*B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *B. cereus* ATCC 14579, *L. acidophilus* ATCC 4356) and gram negative (*E. coli* ATCC 25922, *S. typhymurium* ATCC 14028, *E. aerogenes* ATCC 13048) bacterial strains in culture media. Similar kind of observations were also made by Shahzad et al. (2009) and Ravi et al. (2010) indicating that the essential oil of citrus peel was active against all tested bacteria including both gram positive and negative cultures.

The present investigation for the assessment of antimicrobial activity of *C. limetta* var. Mitha peel oil against different microbes of public health significance indicated a percent decrease in clear zone of inhibition after 96 h ranging between 11 to 27% for different fungi and bacteria. This decrease in clear zone of inhibition after 96 h by sterilized sweet lime peel oil preparation may be either due to inactivation or low concentrations of diffusible water soluble active constituents. Our findings are in agreement with earlier reports (Siddiqui et al., 1996), where a decrease in inhibitory zone from 15 to 26 mm after a period of 96 h of incubation was observed to cause decrease from 9.52 to 34.8% against the tested strains.

Antioxidant activity of *Citrus limetta* var. Mitha peel oil

The ability of essential oils to act as a donor for hydrogen atoms or electrons in the transformation of DPPH radical into its reduced form DPPH-H (which is measured spectrophotometrically) gives them antioxidant activity characteristic. The results of DPPH scavenging activity of *C. limetta* var. Mitha peel oil compared with ascorbic acid as a reference standard are shown in Figure 1 indicating that it has slightly lower antioxidant activity comparative to reference standard, ascorbic acid, being a strong anti-

oxidant reagent. The essential peel oil of *C. limetta* var. Mitha peel oil was able to reduce the stable radical DPPH to yellow-colored DPPH-H reaching 87.77% of DPPH scavenging effect at its 100% concentration whereas the reference standard, ascorbic acid, gave a 99.67% DPPH scavenging effect at its 100% concentration. Mahmud et al. (2009) studies also indicated that essential peel of *C. acida* var. sour lime showed 91.7% of DPPH scavenging effect at its 100% concentration compared with ascorbic acid as a reference standard. Sacchetti et al. (2005) studies also showed that essential oils of *Cananga odorata, Cymbopogon citratus, Rosmarinus officinalis* and *Curcuma longa* notably reduced the concentration of DPPH free radical indicating their strong antioxidant activities. Choi et al. (2000) studies also showed that radical-scavenging activities using DPPH of 31 kinds of citrus essential oils were comparable with or stronger than that of Trolox (standard antioxidant).

In conclusion, the Chemical analysis of essential oil extracted from *C. limetta* var. Mitha (Sweets lime) showed that limonene (95.98%) was found as major component followed by camphene (1.79%), while the remaining terpenes were less than 1%. The results of our study showed that *C. limetta* var. Mitha (sweet lime) peel oil have the probability to be applied as a natural constituent of food preservations, cosmetics and medicines as they exhibit a strong antioxidant, antibacterial and antifungal activity against food borne pathogens.

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